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Description

[0001] This invention relates to cellular nuclear receptors and their uses..

[0002] A large family of nuclear receptors which confer cells with responsiveness to molecules such as retinoid acid, vitamin D, steroid hormones and thyroid hormones has been identified. Extensive studies have shown that the members of this superfamily of nuclear receptors activate and/or repress gene transcription through direct binding to discrete cis-acting elements termed "hormone response elements" (HRE). It has been shown that these HRE's comprise repeats of consensus palindromic hexanucleotide DNA motifs. The specificity of the HRE's is determined by the orientation of, and spacing between, halfsites (i.e. half a palindromic sequence)(Umenesono K., *et al*, 1991 *Cell* **65**, 1255-1265).

[0003] Specific DNA binding is mediated by a strongly-conserved DNA binding domain, containing two zinc fingers, which is conserved among all thus discovered nuclear receptors. Three amino acids at the C-terminal base of the first zinc finger (known as the "P-box") are important for the recognition of the half site nucleotide sequence. Members of the nuclear receptor superfamily have been classified into different groups on the basis of the amino acid sequence within the P box.

[0004] All members of the nuclear receptor superfamily also contain a hypervariable N-terminal domain and a ligand-binding domain containing some "patches" of conserved sequence. One of these is called the "Ti-domain".

[0005] Molecules which are thought to be nuclear receptors, as they are structurally related to characterised receptors, but for which no ligand has been found, are termed "orphan receptors". Many such orphan receptors have been identified (see for example Evans R.M. (1988) *Science* **240**, 889-895 and O'Malley, B. (1990) *Mol. Endocrinol.* **4** 363-369).

[0006] We have now unexpectedly identified, initially in rat a new orphan receptor, which is related to the known estrogen receptor ERα, and which we have designated "ERβ" (specifically "rERβ" in rat). In this specification "Erβ" will be used to refer to the receptors hERβ or rERβ or related receptors. The nucleotide and amino acid sequences of rERβ have now been determined and are shown in Fig. 1. We have also identified a human Erβ - "hERβ", the amino acid DNA and sequences of which are shown in Fig. 13A and 13B respectively.

[0007] According to one aspect of the invention there is provided an estrogen receptor (called ERβ) having the amino acid sequence of Fig. 1, and an estrogen receptor having an amino acid sequence which is more than 95% identical with that sequence; an estrogen receptor (called ERβ) having the amino acid sequence of Fig. 13A, and an estrogen receptor which is more than 89% identical with that amino acid sequence, and which is relatively highly expressed in HUVEC and HOS D4 cells compared to the expression of ERα in those cells and an estrogen receptor (called ERβ) having the amino acid sequence of Fig. 14A and an estrogen receptor having an amino acid sequence which is more than 95% identical with that amino acid sequence. The isolated receptor may be particularly useful in the search for molecules for use in treatment of diseases or conditions such as cardiovascular diseases, central nervous system diseases or conditions or osteoporosis, prostate cancer or benign prostatic hyperplasia.

[0008] The receptor of the invention may also be used in the testing of environmental chemicals for estrogenic activity. There has been increasing concern over the effect of various chemicals released into the environment on the reproduction of humans and animals. Threats to the reproductive capabilities of birds, fish, reptiles, and some mammals have become evident and similar effects in humans have been proposed. Substantial evidence is now emerging which shows that exposure to certain chemicals during critical periods of foetal life may distort the development of the reproductive organs and the immune and nervous systems. On the basis of possible parallels between actual wildlife effects, seen for example in birds and seals living in highly polluted areas, and proposed effects in humans, in combination with documented human reproductive effects caused by prenatal exposure to the pharmaceutical estrogen, diethyl stilbestrol (DES), "estrogenic" chemicals have been proposed to threaten the reproductive capability of both animals and humans. Among the chemicals known or suspected to act as estrogen mimics on the human body, or in other ways disturb the human endocrine system, there are several which have already been identified as environmental hazards. Among the chemicals that have been mentioned as potential causes of disruption of reproductive function in animals and humans are chlorinated organic compounds such dieldrin, endosulfans, chlordanes, endrins, aldrin, DDT and some PCBs, plastics such as Bisphenol A, phthalates and nonylphenol, and aromatic hydrocarbons. Some of the proposed effects on humans have been suggested to be due to an increasing exposure to environmental estrogens - in fact, exposure to chemical compounds to which higher organisms during the foetal period react in a way that is similar to when they are exposed to high dosages of estrogens. The effects are manifested by for example perturbations of the sex characteristics and impaired reproductive potential. In humans, elevated risks of breast cancer and other hormone-related disease has also been discussed as possible effects. In addition, to the documented "estrogenic" effects, it has recently been demonstrated that environmental pollutants may also act on hormonal pathways other than the estrogenic pathway - It has been shown that p,p' - DDE the main metabolite of DDT (also in humans) is a fairly anti-androgenic agent (Kelce W.R. *et al* Nature 1995 **375**:581-585). Epidemiological studies on these issues are, however, presently difficult to interpret. Nevertheless, there is a growing opinion against these potentially hormone disrupting chemicals, and very palpable public and environmental demand for the governmental agencies and industry

to act. In view of the similarities between the receptor of the present invention, Erβ and the classical estrogen receptor, Erβ may be used in the testing of chemicals for estrogenic effect.

[0009] According to another aspect of the invention there is provided a DNA sequence encoding an estrogen receptor according to the first aspect of the invention.

[0010] ERβ is unique in that it is extremely homologous to the rat estrogen receptor, in particular in its DNA binding domain. It appears that ERβ has a very limited tissue distribution. In female rats, it appears to be present only in the ovaries, and in male rats in the prostate and testes. As these tissues are classic targets for estrogen action, it can be deduced that ERβ may mediate some of the effects of estrogen.

[0011] The different ligand specificity of ERα and ERβ may be exploited to design pharmaceutical agents which are selective for either receptor. In particular, the differences in ligand specificity may be used to develop drugs that specifically target cardiovascular disease in postmenopausal women or osteoporosis.

[0012] The nuclear receptor of the invention, ERβ, a method of producing it, and tests on its functionality will now be described, by way of example only, with reference to the accompanying drawings, Figs. 1 to 15 in which:

Fig. 1 shows the amino acid sequence of ERβ and the nucleotide sequence of the gene encoding it;
Fig. 2A is a phylogenetic tree showing the evolution of ERβ and other receptors;
Fig. 2B shows the homology between the different domains in ERβ and certain other receptors:
Fig. 2C is an alignment of the amino acid sequence in the ligand binding domains of rERβ, rERα, mERα and hERα;
Fig. 2D is an alignment of the amino acid sequence in the DNA binding domains of rERβ, rERα, mERα and hERα;
Fig. 3A is a film autoradiograph of prostate gland showing strong expression of a clone of the receptor of the invention, clone 29;
Fig. 3B is a darkfield image showing prominent signal for clone 29 in epithelium (e) of prostatic alveoli. The stroma (s) exhibit(s) weaker signal;
Fig. 3C is a bipolarization image of cresyl violet counterstained section showing silver grains over epithelium (e), whereas the stroma(s) contain(s) less grains;
The bar represents 0.7 mm for Fig. 3A, 200 μm for Fig. 3B and 30 μm for Fig. 3C;
Fig. 4A shows a film autoradiograph of ovary showing strong expression of clone 29 in follicles at different developmental stages (some are indicated by arrows). The interstitial tissue (arrowheads) shows low signal;
Fig. 4B shows a darkfield image showing high expression of clone 29 in granular cells of primary (1), secondary (2), tertiary (3) and mature (4) follicles. Low signal is present in interstitial tissue (it);
Fig. 4C is a bipolarization image of ovary a showing strong signal in granular cells (gc), whereas the oocyte (oc) and the caintema (ti) are devoid of clear signal:
The bar represents 0.9 mm for Fig. 4A, 140 μm for Fig. 4B and 50 μm for Fig. 4C;
Fig. 5A illustrates the results of saturation ligand binding analysis of cloned ERβ;
Fig. 5B illustrates the specificity of ligand binding by cloned ERβ;
Fig. 5C illustrates E2 binding by ERβ;
Fig. 6 illustrates the activation of transcription by cloned ERβ;
Fig. 7 and 7A illustrates stimulation by various ligands by cloned ERβ;
Fig. 8 illustrates the results of RT-PCR experiments on the expression of rat estrogen receptors;
Fig. 9 illustrates the results of RT-PCR experiments on the expression of human Erβ (hERβ);
Fig. 10A is a Hill plot comparing binding of ^{125}I-E2 by hERα and rERβ;
Fig. 10B is a Scatchard plot comparing binding of ^{125}I-E2 by hERα and rERβ;
Fig. 11A illustrates the relative binding affinity of hERα and rERβ for various ligands;
Fig. 11B is a detail of Fig. 12A;
Fig. 12 is an alignment of various estrogen receptors;
Fig. 13A shows the amino acid sequence of human ERβ;
Fig. 13B shows the DNA sequence of human Erβ;
Fig. 14A shows the amino acid sequence of mERβ;
Fig. 14B shows the DNA sequence of mouse Erβ; and
Fig. 15 illustrates ligand binding affinities for various phytoestrogens by ER's of the invention.

A. CLONING OF RAT ERβ

1. PCR-amplification and complementary DNA cloning.

[0013] A set of degenerate primers (DBD 1.2.3 and WAK/FAK) were designed previously according to the most highly conserved sequences of the DNA-binding domain (P-box) and ligand binding domain (Ti-stretch) of members of the nuclear receptor family (Enmark, E., Kainu, T., Pelto-Huikko, M., & Gustafsson, J-A (1994) *Biochem. Biophys. Res.*

Commun. **204**, 49-56). Single strand complementary DNA reverse transcribed from rat prostate total RNA was employed with the primers in PCR reactions as described in Enmark, E., Kainu, T., Pelto-Huikko, M., & Gustafsson. J-Å (1994) *Biochem. Biophys. Res. Commun.* **204**, 49-56. The amplification products were separated on a 2% low melting agarose gel and DNA products between 400 and 700 bp were isolated from the gel and ligated to TA cloning vector (Invitrogen). As alternatives, we also used the RP-I/RP-2 and DBD66-100/DBD210-238 primer sets in the DNA-binding domain of nuclear receptors exactly as described by Hirose T., Fijimoto, W., Yamaai, T., Kim, K.H., Matsuura, H., & Jetten, A.M (1994) *Mol. Endocrinol.* **8**, 1667-1677 and Chang, C., Lopes Da Silva, S., Ideta, R., Lee, Y., Yeh, S., & Burbach, J.P.H (1994) *Proc. Natl. Acad. Sci.* **91**, 6040-6044 respectively. Clone number 29 (obtained with the DBD-WAK/FAK set) with a length of 462 bp showed high homology (65%) with the rat estrogen receptor cDNA (65%), which was previously cloned from rat uterus (Koike, S., Sakai, M., & Muramatsu, M. (1987) *Nucleic Acids Res* **15**, 2499-2513). The amino acid residues predicted by clone 29 DNA sequences suggested that this DNA fragment encoded part of the DNA-binding domain, hinge region and the beginning of the ligand binding domain of a novel member of the nuclear receptor family. Two PCR primers (Figure 1) were used to generate a probe of 204 bp consisting of the hinge region of the novel receptor, which was used to screen a rat prostate cDNA library (Clontech gt10) under stringent conditions resulting in four strongly positive clones with a size of 0.9 kb, 1.8kb, 2.5kb and 5-6kb respectively. The clone of 2.5kb was sequenced and Figure 1 shows the nucleotide sequence determined in the core facility (CyberGene AB) by cycle sequencing using fluorescent terminators (Applied Biosystems) on both strands, with a series of internal primers and deduced amino acid sequence of clone 29. Two in frame ATG codons are located at nucleotide 424 and nucleotide 448, preceding by an in-frame stop codon at nucleotide 319, which suggests that they are possible start codons. The open reading frame encodes a protein of 485 amino acid residues (counted from the first methionine) with a calculated molecular weight of 54.2 kDa. Analysis of the proteins by synthesized by *in-vitro* translation from the clone 29 cRNA in rabbit reticulocyte lysate revealed a doublet protein band migrating at approximately 57 kDa on SDS-PAGE gels (date not shown), confirming the open reading frame. The doublet protein band is probably caused by the use of both ATG codons for initiation of protein synthesis. The amino acid sequence of clone 29 protein shows the characteristic zinc module DNA-binding domain, hinge region and a putative ligand binding domain, which are the characteristic features of members of the nuclear receptor family (Tsai, M-J., & O'Malley, B.W (1994) *Ann. Rev. Biochem.* **63**, 451-486; Härd, T., & Gustafsson, J-Å (1993) *Acc. Chem. Res.* **26**, 644-650; Laudet, V.; Hänni, C., Coll, J., Catzeflis, F., & Stehelin, D (1992) *EMBL J.* **11**, 1003- 1012).

[0014] Protein sequence comparison with several representative members of the nuclear receptor family (Figure 2) showed the clone 29 protein is most related to the rat estrogen receptor (ERα), cloned from uterus (Koike, S., Sakai, M., & Muramatsu, M. (1987) *Nucleic. Acids Res.* **15**, 2499-2513), with 95% identity in the DNA-binding domain (amino acid residues 103-167) (Griffiths, K., Davies, P., Eaton, C.I., Harper, M.E., Turkes, A., & Peeling, W.B. (1991) in *Endocrine Dependent Tumours*, eds. Voigt, K-D. & Knabbe, C. (Raven Press), pp. 83-125). A number of functional characteristics have been identified within the DNA-binding domain of nuclear receptors (Hard, T., & Gustafsson, J-Å. (1993) *Acc. Chem. Res 26*, 644-650 and Zilliacus, J., Carlstedt-Duke, J., Gustafsson, J-A., & Wright, A.P.H. (1994) *Proc. Natl. Acad. Sci. USA* 91, 4175-4179). The so-called P-box specifies nucleotide sequence recognition of the core half-site within the response element, while the D- box mediates dimerization between receptor monomers. The clone 29 protein P-box and D-box sequences of EGCKA and PATNQ, respectively, are identical to the corresponding boxes in ERα (Härd, T., & Gustafsson, J-Å, *(1993) Acc. Chem. Res* **26**, 644-650 and Koike. S., Sakai, M., & Muramatsu, M. (1987) *Nucleic Acids Res.* **15**, 2499-2513), thus predicting that clone 29 protein binds to ERE sequences.

[0015] The putative ligand binding domain (LBD) of clone 29 protein (amino acid residues 259-457) shows closest homology to the LBD of the rat ERα (Figure 2), while the homology with the human ERR1 and ERR2 proteins (Giguere, V., Yang, N., Segui, P., & Evans R.M. (1988) *Nature* 331, 91-94) is considerably less. With the human, mouse and xenopus estrogen receptors the homology in the LBD is also around 55%, while the homology with the LBD of other steroid receptors is not significant (Figure 2). Cysteine residue 530 in human ERα has been identified as the covalent attachment site of an estrogenic affinity label (Harlow, K.W., Smith D.N., Katzenellenbogen, J.A., Greene, G.L., & Katzenellenbogen, B.S. (1989) *J. Biol. Chem.* **264**, 17476- 17485). Interestingly, clone 29 protein (Cys-436) as well as the mouse, rat and xenopus ERas have a cysteine residue at the corresponding position. Also, two other amino acid residues described to be close to or part of the ligand-binding pocket of the human ERα-LBD (Asp 426 and Gly 521) are conserved in the LBD of clone 29 protein (Asp 333 and Gly 427) and in the LBD of ERas from various species (20.21). The ligand-dependent transactivation function TAF-2 identified in ERα (Danielian, P.S., White, R., Lees, J.A., & Parker, M.G. (1992) *EMBO J.* **11**, 1025-1033), which is believed to be involved in contacting other transcription factors and thereby influencing activation of transcription of tarteg genes, is almost completely conserved in clone 29 protein (amino acid residues 441-457). Steroid hormone receptors are phosphoproteins (Kuiper. G., & Brinkmann, A. O. *(1994) Mol. Cell. Endocrinol.* **100**, 103-107), and several phosphorylation sites identified in the N-terminal domain and LBD of ERα (Arnold, S.F., Obourn, J.D., Jaffe, H., & Notides, A.C. (1995) *Mol. Endocrinol.* **9**, 24-33 and Le Goff, P., Montano, M.M., Schodin, D.J., & Katzenellenbogen, B.S (1994) J. *Biol. Chem.* **269**, 4458-4466) are conserved in clone 29 protein (Ser 30 and 42, Tyr 443). Clone 29 protein consists of 485 amino acid residues while ERαs from

human, mouse and rat consist of 590-600 amino acid residues. The main difference is a much shorter N-terminal domain in clone 29 protein i.e 103 amino acid residues as compared to 185-190 amino acid residues in the other receptor proteins. Also the non-conserved so-called F-domain at the C-terminal end of ERas is 15 amino acid residues shorter in clone 29 protein. The cDNA insert of a positive clone of 2.6 kb was subcloned into the EcoR1 site of pBluescript (trademark) (Stratagene). The complete DNA sequence of clone 29 was determined (CyberGene AB) by cycle sequencing using fluorescent terminators (Applied Biosystems) on both strands. with a series of internal primers.

[0016] Figs 2C and 2D respectively compare the ligand and DNA binding domain of Erβ compared to rat, mouse and human Era's.

2. **Saturation ligand binding analysis and ligand competition studies:**

[0017] Clone 29 cDNA was subcloned in pBluescript downstream of the T7 promoter to give p29-T7. Clone 29 protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega). Translation reaction mixtures were diluted five times with TEDGMo buffer (40 mm Tris/HCl, pH 7.4, 1mM EDTA, 10% (v/v) glycerol, 10 mM Na_2MoO_4, 10 mM DTT) and 0.1 ml aliquots were incubated for 16 h at 8° C with 0.3- 6.2 nM [2,4,6,7-^{3}H]-17β-estradiol (NEN-Dupont; specific radioactivity 85 Ci/mmol) in the presence or absence of a 200-fold excess of unlabelled E2.

[0018] Fig. 5A illustrates the results of a saturation ligand analysis of clone 29 protein. Reticulocyte lysate containing clone 29 protein was incubated with 6 concentrations of [^{3}H]E2 between 0.3 and 6.0 nM. Parallel tubes contained an additional 200 fold of non-radioactive E2. Bound and free ligand were separated with a dextran-coated charcoal assay. The Kd (0.6 nM) was calculated from the slope of the line in the Scatchard plot shown (r = 0.93), and the number of binding sites was extrapolated from the intercept on the abscissa (Bmax = 1400 fmol/ml undiluted translation mixture).

[0019] For ligand competition studies diluted reticulocyte lysate was incubated with 5 nM [2,4,6,7-^{3}H]-17β-estradiol in the presence of either 0, 5, 50, 500 or 5,000 nM of non- radioactive E2, estrone, estriol, testosterone, progesterone, corticosterone, 5α-androstane-3β,17β-diol, 5α-androstanc-3α,17β-diol and diethylstilbestrol (DCES) for 16 h at 8°C. Bound and unbound steroids were separated with a dextran-coated charcoal assay (Ekman, P., Barrack, E.R., Greene, G.L., Jensen, E.V., & Walsh, P.C (1983) J. Clin. *Endocrinol Metab.* **57**, 166-176).

[0020] Fig. 5B illustrates the specificity of ligand binding by clone 29 protein. Reticulocyte lysate containing clone 29 protein was equilibrated for 16 h with 5 nM [^{3}H]E2 and the indicated fold excess of competitors. Data represent [^{3}H] E2 bound in the presence of unlabelled E2. testosterone (T), progesterone (prog), corticosterone (cortico), estrone (E1), diethylstilbestrol (DES), 5α-androstane-3α, 17β-diol (3α-AD), 5α-androstane-3β,17β-diol (3β-AD) and estriol (E3). [^{3}H]E2 binding in the absence of competitor was set at 100%.

3. ***In-situ* hybridisation:**

[0021] *In-situ* hybridisation was carried out as previously described (Dagerlind Å., Friberg, K., Bean. A.J., & Hökfelt, T (1992) *Histochemistry* **98**, 39-49). Briefly, two oligonucleotide probes directed against nucleotides 994-1041 and 1981-2031 were each labelled at the 3'-end with ^{33}P-dATP using terminal deoxynucleotidyltransferase (Amersham, UK). Adult male and female Sprague-Dawley rats (age 2 to 3 months n=10) were used for this study. The rats were decapitated and the tissues were rapidly excised and frozen on dry ice. The tissues were sectioned in a Microm HM500 cryostat at 14 µm and thawed onto Probe-On glass slides (Fisher Scientific, PA, USA). The slides were stored at -20°C until used. The slides were incubated in humidified boxes at 42°C for 18 h with 1x10^6 cpm ol the probe in a hybridization solution containing 50% formamide, 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 x Denhardt (0.02 % BSA, 0.02 % Ficoll, 0.02 % PVP), 1 % sarkosyl, 0.02 M sodium phosphate (pH 7.), 10% dextransulphate, 500 µg/ml salmon sperm DNA and 200 mM DTT. Slides were subsequently rinsed in l x SSC at 55°C for 60 min with four changes of SSC and finally in 1 x SSC starting at 55°C and slowly cooled to room temperature, transferred through distilled water and briefly dehydrated in 50% and 95% ethanol for 30 sec each, air-dried, and covered with Amersham β-man autoradiography film for 15 to 30 days. Alternatively the slides were dipped in Kodak NTB2 nuclear track emulsion (diluted 1:1 with distilled water) and exposed for 30 to 60 days at 4°C. Finally, the sections were stained with cresyl violet.

[0022] Clear expression of clone 29 was observed in the reproductive tract of both male and female rats, while in all other rat tissues the expression was very low or below the level of detection with *in-situ* hybridisation (not shown). In male reproductive organs high expression was seen in the prostate gland (Figure 3), while very low expression was observed in testis, epididymis and vesicula seminalis (not shown). In dipped sections, expression was clearly visible in prostate epithelial cells (secreting alveoli) while the expression in smooth muscle cells and fibroblasts in the stroma was low (Figure 3). In female reproductive organs expression was seen in the ovary (Figure 4), while uterus and vagina were negative (not shown). In dipped sections high expression was seen in the granulosa cell layer of primary, secondary and mature follicles (Figure 4), whereas primordial follicles, oocytes and corpora lutea appeared completely negative. Low expression was seen in the interstitial cells of the ovary. Both anti-sense oligonucleotide probes used produced similar results. Addition of a 100 fold excess of the respective unlabelled oligonucleotide probes during the

hybridisation reactions abolished all signals.

4. **Transactivation analysis in CHO-cells:**

[0023] The expression vector pCMV29 was constructed by inserting the 2.6 kb clone 29 tragment in the EcoRI site of the expression vector pCMV5 (Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., & Russell, D.W. (1989) *J. Biol. Chem.* **264**, 8222-8229). The pERE-ALP reporter construct contains a secreted torm ol the plancental alkaline phosphatase gene (Berger, J., Hauber, J., Hauber, R., Geiger, R., & Cullen, B.R. (1988) *Gene* **66**, 1-10) and the MMTV-LTR in which the glucocorticoid response elements were replaced by the vitellogenin promoter estrogen response element (ERE).

[0024] CHO-K1 cells were seeded in 12-well plates at approximately 1.7×10^5 cells per well in phenol-red free Ham F12 medium with 5% FCS (dextran-coated charcoal treated) and 2 mM Lglutamine. After 24 h the cells were transfected with 250 ng pERE-ALP vector and 50 ng pCMV29 using lipofectamine (Gibco) according to the manufacturer's instructions. After five hours ot incubation the cells were washed and refed with 0.5 ml phenol-red free Coon's F-12 medium containing 5% serum substitute (SRC 3000, Tissue Culture Services Ltd., Botolph Claydon, Buckingham, UK) 2 mM Lglutamine and 50 µg/ml gentamicin plus hormones as indicated. After 48 h the medium was assayed for alkaline phosphatase (ALP) activity by a chemiluminescence assay. A 10 µl aliquot of the cell culture medium was mixed with 200 µl assay buffer (10 mM diethanolamine pH 10.0 1 mM $MgCl_2$ and 0.5 mM CSPD (Tropix Inc. Boston, USA)) and incubated for 20 min at 37°C before measurement in a microplate luminometer (Luminoskan; Labsystems, Finland) with integral measurement for 1 second. The ALP activity of ERE-reporter alone was set at 1.

5. Ligand binding characteristics and transactivation function of clone 29 protein:

[0025] On the basis of the described high homology between clone 29 protein and rat ERα in the DBD and LBD it was hypothesized that clone 29 protein might encode a novel ER. Furthermore, biological effects of estrogens on rat prostate and ovary, which show high expression of clone 29 RNA, are well known (Griffiths, K., Davies, P., Eaton, C. I., Harper, M.E., Turkes, A., & Peeling W. B. (1991) in *Endocrine Dependent Tumours*, eds Voigt, K-D. & Knabbe, C. (Raven Press), pp 83-125; Richards, J.S (1994) *Endocrine Rev.* **15**, 725-745; and Habenicht, U-F., Tunn, U.W., Senge, Th., Schroder, R.H., Schweikert, H.U., Bartsch, G., & El Etreby, M.F. (1993) *J. Steroid Biochem. Molec. Biol.* **44**, 557-563). In order to analyze the steroid binding properties of clone 29 protein synthesized *in vitro*, the reticulocyte lysate was incubated at 8°C for 16 h with increasing concentrations (0.3-6.0 nM) of [^{3}H]E2 in the presence or absence of a 200 fold molar excess of unlabelled E2. Linear transformation of saturation data revealed a single population of binding sites for E2 with a K_d (dissociation constant) of 0.6 nM (Figure 5A and C). Steroid binding specificity was measured by incubating reticulocyte lysate with 5 nM [^{3}H]E2 in the presence of 0.5, 50, 500 and 5,000 nM unlabelled competitors. Competition curves generated are indicative of an estrogen receptor in that only estrogens competed efficiently with [^{3}H]E2 for binding (Figure 5B). Fifty percent inhibition of specific binding occured by 0.6 fold excess of unlabelled E2; diethylstilbestrol, estriol. estrone and 5α-androstane-3β,17β-diol were 5, 15, 50 and 150 times, respectively, less effective as competitors. Neither testosterone, progesterone. corticosterone nor 5α- androstane-3α,17β-diol were efficient competitors, even at the highest concentrations used (1000 told excess). The dissociation constant and the steroid binding specificities measured are in good agreement with data previously reported for ERs in rat and human prostate, rat granulosa cells. rat antral follicles and whole rat ovarian tissue (Ekman, P., Barrack, E.R., Greene, G.L., Jensen, E.V., & Walsh, P.C (1983) J. *Clin. Endocrinol. Metab.* **57**, 166-176; van Beurden-Lamers, W.M.O., Brinkmann, A-O., Mulder, E., & van der Molen, H. (1974) *Biochem. J* **140**, 495-502; Kudolo, G.B., Elder, M.G., & Myatt, L. (1984) J. *Endocrinol.* **102**, 83-91; and Kawashima, M., & Greenwald, G.S. (1993) *Biology of Reprod.* **48** 172-179).

[0026] When clone 29 protein was labelled with a saturating dose of [^{3}H]E2 and analyzed on sucrose density gradients, a single peak of specifically bound radioactivity was observed. The sedimentation coefficient of this complex was about 7S, and it shifted to 4S in the presence of 0.4 M NaCl (not shown). To investigate the transcriptional regulatory properties of clone 29 protein, we performed co-transfection experiments in which CHO cells were transfected with a clone 29 protein expression vector and/or an estrogen-responsive reporter gene construct. Cells were incubated in the absence of E2 (clone 29) or in the presence of 100 nM E2 (Clone 29 + E2) or in the presence of 100 nM E2 and 12 µM Tamoxifen (Clone 29 + E2/Tam). In the absence of exogenously added E2 clone 29 protein showed considerable transcriptional activity which could be further increased by the addition of 100 nM E2 (Figure 5). Simultaneous addition of a 10 fold excess of the antiestrogen Tamoxifen partially suppressed the E2 stimulated activity (Figure 6). The constitutive transcriptional activity of clone 29 protein could be suppressed by the anti-estrogen ICI-1624384 (not shown).

It has been shown previously that the wild-type mouse and human ERs are constitutive activators of transcription, and that the transcriptional activity can be stimulated further by the addition of E2 (Tzukerman, M., Xiao-Kun Zhang., Hermann, T., Wills, K. N., Graupner, G., & Phal, M, (1990) *New Biologist* **2**, 613-620 and Lees, J.A., Fawell, S.E., & Parker, M.G. (1989) *Nucl. Acids Res.* **17**, 5477-5488). To obtain more insight into what concentrations of E2 effect clone 29

protein transcriptional activity, transient transfection experiments were carried out in the presence of increasing concentrations of E2. CHO-cells were transiently transfected with the ERE-reporter plasmid and the clone 29 protein expression plasmid. Cells were incubated with increasing concentrations of E2 (0.1 - 1000 nM), estrone (E1, 1000 nM), 5α-androstane-3β,17β-diol (3β-AD, 1000 nM) or no ligand added. Alkaline phosphatase activity (ALP) was measured as described and the activity in the absence of ligand (control) was set at 1. The figure shows relative ALP-activities (=SD) from three independent experiments. Clone 29 protein began to respond at 0.1 nM E2 and maximal stimulation was observed between 1 nm and 10 nM E2 (Figure 7). The maximal stimulation factor was 2.6 ± 0.5 fold (mean = SD, n = 9) as compared to incubation in the absence of E2. Apart from E2 also estrone and 5α-androstane-3β,17β-diol could stimulate transcriptional activity, albeit at higher concentrations (Figure 7). Dexamethasone, testosterone, progesterone, 5α-androstane-3α,17β-diol, thyroid hormone and *all-trans*-retinoic acid could not stimulate transcriptional activity of clone 29 protein, even at the highest concentration (1000 nM) tested (not shown). The results of the co-transfection experiments are in agreement with the ligand binding and specificity data of clone 29 protein presented in Figure 5. In control experiments, wild-type human ERα also showed transcriptional activity in the absence of E2, which could be increased by the addition of E2 (not shown).

6. Detection of rat ER expression by RT-PCR

[0027] The tissue specificity of expression of rat ERβ and ERα was determined using reverse transcriptase polymerase chain reaction (RT-PCR). The results of the experiment are shown in Fig. 8.

B. Isolation of human Erβ

[0028] 1. A human version of Erβ (hERβ) has also been cloned from human ovary. The tissue specificity of hERβ expression in a variety of cells was also determined using the RT-PCR technique. The results are shown in Fig. 9. It will be noticed that there is a very high level of mRNA of hERβ in human umbilical vein endothelial cells (HUVEC) but no detection of hERα in the same cells. In addition, it will be seen that in human osteosarcoma cell line (HOS-D4), hERβ is expressed in greater quantities compared to hERα.

[0029] I. A human version of ERβ (hERβ) has also been cloned. The tissue specificity of hERβ expression in a variety of cells was also determined using the RT-PCR technique. The results are shown in Fig. 9. It will be noticed that there is a very high level of mRNA of hERβ in human umbilical vein endothelial cells (HUVEC) but no detection of hERα in the same cells. In addition, it will be seen that in human osteosarcoma cell line (HOS-D4), hERβ is expressed in greater quantities compared to hERα.

[0030] The partial DNA sequence of hERβ is shown in Fig. 10 and a derived amino acid sequence is shown in Fig. 11.

Cloning of human Erβ from testis

[0031] A commercially available cDNA from human testis (Clontech, article no. HL1161x) was screened, using a fragment containing the ligand-binding domain of the rat Erβ cDNA as probe. Approximately 10^6 recombinants were screened, resulting in one positive clone. Upon sequencing of this clone, it was seen that the insert was 1156 bp (Figure 13A and 13B). This corresponds to most of the translated region of a receptor with an overall homology of 90.0% to rat Erβ, therefore deduced to represent the human form of Erβ.

[0032] The cloned hERβ, however, lacks approximately 47 amino acids at the N-terminal end and 61 amino acids at the C- terminal end (as compared to the rat sequence). Further screening of the same library was unsuccessful. PCR technology was therefore used to obtain the remaining parts. For oligonucleotides were synthesised; two degenerate oligonucleotides containing all possible codons for the amino acids adjacent to the initiation methionine and the stop codon, respectively, of the rat Erβ, and two specific oligonucleotides containing the sequence of the clone isolated from the human testis library and situated approximately 100 bp from respective end of this clone. PCR with the N-terminal and C-terminal pair of oligos yielded specific bands, that were subcloned and sequenced. The parts of these new clones that overlap the original cDNA clone are identical to this. It was thus possible to construct peptide and DNA sequences corresponding to the whole open reading frame (Fig. 13A and 13B).

[0033] When comparing the human Erβ to rat Erβ, this receptor is 79.6% identical in the N-terminal domain, 98.5% in the DNA-binding domain, 85.6% in the hinge and 91.6% in the ligand-binding and F-domains. These numbers match very well those found when comparing the rat and human forms of Era.

[0034] Studies of the expression of human Erβ using Northern blot show expression in testis and in ovaries. The expression in prostate, however, appears lower than found in the rat.

[0035] The human Erβ gene has been mapped to chromosome 14 using PCR and to region 14q22-23 using the FISH technique, whereas the human Erβ gene has been mapped to chromosome 6q25.

2. Comparison of ligand binding affinity of hERα and rERβ

[0036] The ligand affinity of the two estrogen receptors, human Erα (ovary) (hERα) and rat Erβ (rERβ) was tested in binding saturation experiments and in binding competition experiments.

[0037] cDNA of the receptor subtypes hERα and rERβ were *in vitro* translated in rabbit reticulocyte lysate in presence of non-radioactive amino acids according to the instructions supplied by the manufacturer (Promega).

[0038] The radioactive ligand used in all experiments was 16α-[^{125}I]-17β-estradiol ([^{125}I]-E2) (NEX- 144, New England Nuclear). The method for the binding experiments was previously described in: Salomonsson M, Carlsson B, Haggblad J. J. *Steroid Biochem. Molec. Biol.* Vol. 50, No. 5/6 pp. 313-18, 1994. In brief, estrogen receptors are incubated with [^{125}I]-E2 to equilibrium (16-18 h at +4°C). The incubation was stopped by separation of protein-bound [^{125}I]-E2 from free [^{125}I]-E2 on Sephadex G25 columns. The radioactivity of the eluate is measured in a gamma-counter.

[0039] In the competition experiments, non-radioactive ligands were diluted in DMSO, mixed with [^{125}I]-E2 (approximately 100-200 pM), aliquoted in parallel, and finally hERα or rERβ was added. The final concentration of DMSO in the binding buffer was 2%.

[0040] The buffer used in the experiments was of the following composition: Hepes (pH=7.5) 20 mM, KCl 150 mM, EDTA 1 mM, glycerol (8.7%), monothioglycerol 6 mM, $Na_3MO_4$10mM.

3. Equilibrium binding saturation experiments (K_d-determinations)

[0041] A range of concentrations of [^{125}I]-E2 were mixed with the ER:s and incubated as described above, free [^{125}I]-E2 was determined by substracting bound [^{125}I]-E2 from added [^{125}I]-E2. Binding data was analysed by Hill-plots and by Scatchard plots (Figure 11). The equilibrium binding results are shown in Table 1. The apparent K_d-values for [^{125}I]-E2 differed between the two ER:s with approximately a factor of four: K_d(hERα):K_d(rERβ) = 1:4.

Table 1.

Equilibrium dissociation constants for [^{125}I]-E2 to the two subtypes.		
Receptor subtype	K_d (Hill-plot)	K_d (Scatchard-plot)
hERα	0.06 nM	0.09 nM
rERβ	0.24 nM	0.42 nM

4. Competition experiments (IC_{50} determinations)

[0042] The experiments were performed as described above. IC_{50} values were obtained by applying a four parameter logistic analysis; $b=((b_{max}-b_{min})/(1+(I/IC_{50})^S))+b_{min}$, where I is the added concentration of binding inhibitor, IC_{50} is the concentration of inhibitor at half maximal binding and S is a slope factor. The free concentration of [^{125}I]-E2 was determined by sampling an aliquot from the wells at the end of the incubation and then substract bound radioactivity from sampled total radioactivity.

[0043] Since the equilibrium binding experiments (above) showed that the K_d-values for [^{125}I]-E2 differed between the two ER:s, K_i-values (from the Cheng-Prusoff equation: $K_i=IC_{50}/(1+L/K_d)$ where L is free ([^{125}I]-E2]) were calculated for the compounds investigated. Two approaches for calculating RBA (Relative Binding Affinity) were used. The RBA values were derived using either the IC_{50} values or the K_i values. In both approaches, the value for the compound 16α-bromo-estradiol was selected as the reference value (100%). Both approaches gave similar results. The results are summarized in Figure 12. In these Figures "4-OH-Tam" = 4-hydroxy-tamoxifen; "DES" = diethylstilbestrol; "Hexestr" = hexestrol; "ICI-164384" = ICI plc compound no. 164382; "17β-E2" = 17β-estradiol; "16a-B- E2" = 16α-bromo-estradiol; "Ralox" = Raloxifen; and "17a-E2" 17α diol.

[0044] The results show that Erα and Erβ have significant different ligand binding affinities - the apparent K_d-values for [^{125}I]-E2 differed between the two ER's by a factor of about 4 (K_d(hERα): K_d (rERβ) ≈ 1:4). Some compounds investigated showed significant differences in the competition for binding of [^{125}I]-E2 to the ER's. Certain compounds were found to be more potent inhibitors of [^{125}I]-E2 binding to hERα as compared to rERβ whereas others were found to be more potent inhibitors of [^{125}I]-E2 binding to rERβ than to hERα.

Claims

1. An isolated estrogen receptor (called ERβ) having the amino acid sequence of Fig. 1.

2. An isolated estrogen receptor (called ERβ) having an amino acid sequence which is more than 95% identical to the amino acid sequence of Fig. 1.

3. An isolated estrogen receptor (called ERβ) having the amino acid sequence of Fig. 13A.

4. An isolated estrogen receptor (called ERβ) having an amino acid sequence which is more than 89% identical to the amino acid sequence of Fig. 13A, and which is expressed in greater quantities in HUVEC and HOS D4 cells than ERα.

5. An isolated estrogen receptor (called ERβ) having the amino acid sequence of Fig. 14A.

6. An isolated estrogen receptor (called ERβ) having an amino acid sequence which is more than 95% identical to the amino acid sequence of Fig. 14A.

7. An estrogen receptor according to any one of claims 1 to 6 which is derived from mammalian cells.

8. An estrogen receptor according to claim 7 which is derived from rat or human cells.

9. An isolated DNA sequence encoding an estrogen receptor according to any preceding claim.

10. An isolated DNA sequence according to claim 9 in which the DNA sequence is one of those given in Figs.1, 13B or 14B.

11. An isolated nucleic acid sequence which is capable of hybridizing under stringent conditions to an oligonucleotide probe comprising nucleotides 994-1041 of Fig. 1, wherein said nucleic acid sequence codes for an estrogen receptor according to any one of claims 1 to 8.

12. An isolated nucleic acid sequence which is capable of hybridizing under stringent conditions to an oligonucleotide probe comprising nucleotides 1981-2031 of Fig.1, wherein said nucleic acid sequence codes for an estrogen receptor according to any one of claims 1 to 8.

13. The use of an estrogen receptor according to any one of claims 1 to 8, or a DNA sequence according to claim 9 or 10, or a nucleic acid sequence according to claim 11 or 12 in an assay to determine molecules which bind an estrogen receptor according to any one of claims 1 to 8.

14. The use of an estrogen receptor according to any one of claims 1 to 8, or a DNA sequence according to claim 9 or 10, or a nucleic acid sequence according to claim 11 or 12 in an assay to determine molecules for use in the treatment of ERβ specific diseases or conditions or diseases or conditions related to an estrogen receptor according to any one of claims 1 to 8.

15. The use of an estrogen receptor according to any one of claims 1 to 8, or a DNA sequence according to claim 9 or 10, or a nucleic acid sequence according to claim 11 or 12 in an assay to determine molecules for use in the treatment of prostate or ovarian cancer, benign prostatic hyperplasia, diseases of the central nervous system, osteoporosis, or cardiovascular disease.

16. A drug design method comprising comparing binding of a test compound to ERa and to an estrogen receptor according to any one of claims 1 to 8.

17. The use of an estrogen receptor according to any one of claims 1 to 8 in the testing of the possible estrogenic or other hormonal effects of a substance.

Patentansprüche

1. Isolierter Östrogenrezeptor (benannt ERβ) mit der Aminosäuresequenz von Fig. 1.

2. Isolierter Östrogenrezeptor (benannt ERβ) mit einer Aminosäuresequenz, die zu mehr als 95 % identisch ist mit der Aminosäuresequenz von Fig. 1.

3. Isolierter Östrogenrezeptor (benannt ERβ) mit der Aminosäuresequenz von Fig. 13A.

4. Isolierter Östrogenrezeptor (benannt ERβ) mit einer Aminosäuresequenz, die zu mehr als 89 % identisch ist mit der Aminosäuresequenz von Fig. 13A und die in HUVEC- und HOS D4-Zellen in größeren Mengen als ERa exprimiert wird.

5. Isolierter Östrogenrezeptor (benannt ERβ) mit der Aminosäuresequenz von Fig. 14A.

6. Isolierter Östrogenrezeptor (benannt ERβ) mit einer Aminosäuresequenz, die zu mehr als 95 % identisch ist mit der Aminosäuresequenz von Fig. 14A.

7. Östrogenrezeptor nach einem der Ansprüche 1 bis 6, der von Säugerzellen stammt.

8. Östrogenrezeptor nach Anspruch 7, der von Ratten- oder menschlichen Zellen stammt.

9. Isolierte DNA-Sequenz, die einen Östrogenrezeptor nach einem der vorangehenden Ansprüche codiert.

10. Isolierte DNA-Sequenz nach Anspruch 9, in der die DNA-Sequenz eine der Sequenzen ist, die in den Figuren 1, 13B oder 14B gezeigt ist.

11. Isolierte Nucleinsäuresequenz, die unter stringenten Bedingungen mit einer Oligonucleotidsonde hybridisieren kann, die die Nucleotide 994-1041 von Fig. 1 umfaßt, wobei die Nucleinsäuresequenz einen Östrogenrezeptor nach einem der Ansprüche 1 bis 8 codiert.

12. Isolierte Nucleinsäuresequenz, die unter stringenten Bedingungen mit einer Oligonucleotidsonde hybridisieren kann, die die Nucleotide 1981-2031 von Fig. 1 umfaßt, wobei die Nucleinsäuresequenz einen Östrogenrezeptor nach einem der Ansprüche 1 bis 8 codiert.

13. Verwendung eines Östrogenrezeptors nach einem der Ansprüche 1 bis 8 oder einer DNA-Sequenz nach Anspruch 9 oder 10 oder einer Nucleinsäuresequenz nach Anspruch 11 oder 12 in einem Test zur Bestimmung von Molekülen, die an einen Östrogenrezeptor nach einem der Ansprüche 1 bis 8 binden.

14. Verwendung eines Östrogenrezeptors nach einem der Ansprüche 1 bis 8 oder einer DNA-Sequenz nach Anspruch 9 oder 10 oder einer Nucleinsäuresequenz nach Anspruch 11 oder 12 in einem Test zur Bestimmung von Molekülen zur Verwendung in der Behandlung von ERβ-spezifischen Krankheiten oder Zuständen oder Krankheiten oder Zuständen, die mit einem Östrogenrezeptor nach einem der Ansprüche 1 bis 8 im Zusammenhang stehen.

15. Verwendung eines Östrogenrezeptors nach einem der Ansprüche 1 bis 8 oder einer DNA-Sequenz nach Anspruch 9 oder 10 oder einer Nucleinsäuresequenz nach Anspruch 11 oder 12 in einem Test zur Bestimmung von Molekülen zur Verwendung in der Behandlung von Prostata- oder Eierstockkrebs, gutartiger Prostatahyperplasie, Krankheiten des zentralen Nervensystems, Osteoporose oder Herz-Kreislauf-Erkrankungen.

16. Verfahren zur Konstruktion eines Wirkstoffes, umfassend das Vergleichen der Bindung einer Testverbindung an ERa und an einen Östrogenrezeptor nach einem der Ansprüche 1 bis 8.

17. Verwendung eines Östrogenrezeptors nach einem der Ansprüche 1 bis 8 zum Testen der möglichen Östrogen- oder anderer hormonaler Wirkungen eines Stoffes.

Revendications

1. Récepteur d'estrogènes isolé (appelé ERβ) ayant la séquence d'aminoacides de la figure 1.

2. Récepteur d'estrogènes isolé (appelé ERβ) ayant une séquence d'aminoacides identique à plus de 95 % à la séquence d'aminoacides de la figure 1.

3. Récepteur d'estrogènes isolé (appelé ERβ) ayant la séquence d'aminoacides de la figure 13A.

4. Récepteur d'estrogènes isolé (appelé ERβ) ayant une séquence d'aminoacides identique à plus de 89 % à la séquence d'aminoacides de la figure 13A, et qui est exprimé en de plus grandes quantités dans les cellules HUVEC (cellules endothéliales de la veine ombilicale humaine) et HOS-D4 (lignée cellulaire d'ostéosarcome humain) que l'ERα.

5. Récepteur d'estrogènes isolé (appelé ERβ) ayant la séquence d'aminoacides de la figure 14A.

6. Récepteur d'estrogènes isolé (appelé ERβ) ayant une séquence d'aminoacides identique à plus de 95 % à la séquence d'aminoacides de la figure 14A.

7. Récepteur d'estrogènes selon l'une quelconque des revendications 1 à 6, qui est dérivé de cellules de mammifères.

8. Récepteur d'estrogènes selon la revendication 7, qui est dérivé de cellules de rat ou de cellules humaines.

9. Séquence d'ADN isolée codant pour un récepteur d'estrogènes selon l'une quelconque des revendications précédentes.

10. Séquence d'ADN isolée selon la revendication 9, dans laquelle la séquence d'ADN est l'une de celles données dans les figures 1, 13B ou 14B.

11. Séquence d'acides nucléiques isolée, qui est capable de s'hybrider dans des conditions stringentes à une sonde oligonucléotidique comprenant les nucléotides 994-1041 de la figure 1, ladite séquence d'acides nucléiques codant pour un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8.

12. Séquence d'acides nucléiques isolée, qui est capable de s'hybrider dans des conditions stringentes à une sonde oligonucléotidique comprenant les nucléotides 1981-2031 de la figure 1, ladite séquence d'acides nucléiques codant pour un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8.

13. Utilisation d'un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8, ou d'une séquence d'ADN selon la revendication 9 ou 10, ou d'une séquence d'acides nucléiques selon la revendication 11 ou 12, dans une analyse destinée à la détermination de molécules qui se lient à un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8.

14. Utilisation d'un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8, ou d'une séquence d'ADN selon la revendication 9 ou 10, ou d'une séquence d'acides nucléiques selon la revendication 11 ou 12, dans une analyse destinée à la détermination de molécules à utiliser dans le traitement de maladies ou de troubles spécifiques de l'ERβ ou de maladies ou de troubles associés à un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8.

15. Utilisation d'un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8, ou d'une séquence d'ADN selon la revendication 9 ou 10, ou d'une séquence d'acides nucléiques selon la revendication 11 ou 12, dans une analyse destinée à la détermination de molécules à utiliser dans le traitement du cancer de la prostate ou de l'ovaire, de l'adénome prostatique, de maladies du système nerveux central, de l'ostéoporose ou de maladies cardio-vasculaires.

16. Procédé de conception de médicaments comprenant la comparaison de la liaison d'un composé d'essai à l'ERa et à un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8.

17. Utilisation d'un récepteur d'estrogènes selon l'une quelconque des revendications 1 à 8 dans la recherche d'éventuels effets estrogènes ou d'autres effets hormonaux d'une substance.

FIG. 1

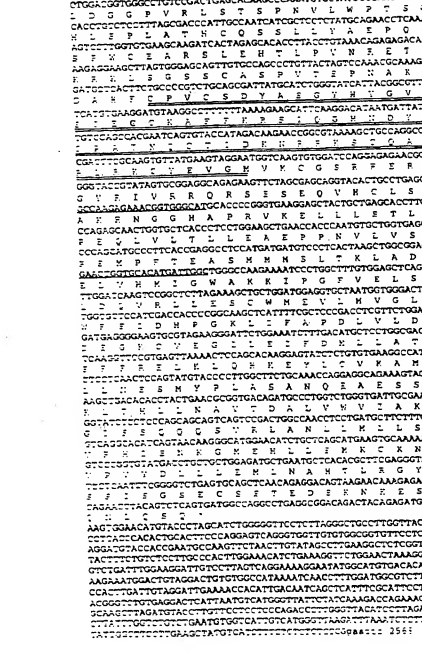


FIG. 2A

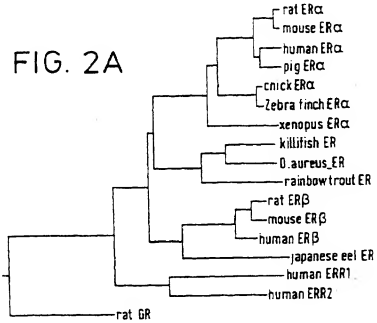
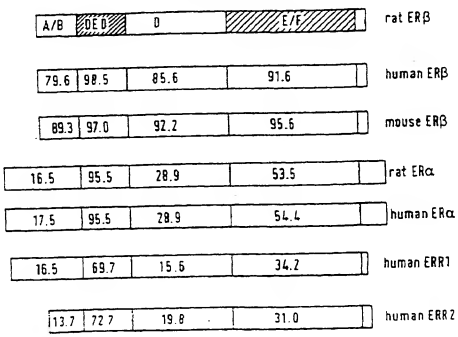


FIG. 2B

ALIGNMENT OF ERβ TO OTHER ESTROGEN RECEPTORS



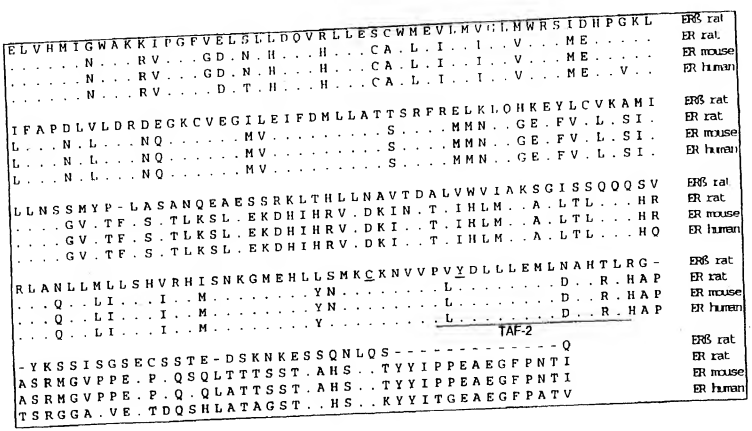


FIG. 2C

DNA-binding domain

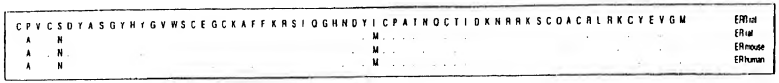


FIG. 2D

FIG. 3A

FIG. 3B

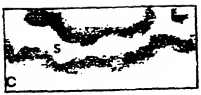


FIG. 3C

FIG. 4A

FIG. 4B

FIG. 4C

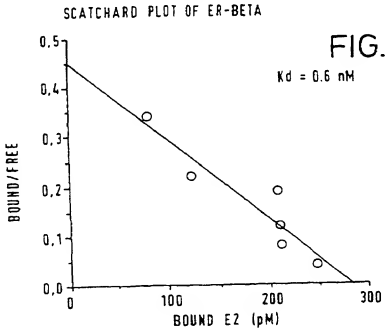

SCATCHARD PLOT OF ER-BETA
Kd = 0.6 nM
BOUND/FREE
0,5
0,4
0,3
0,2
0,1
0,0
0
100
200
300
BOUND E2 (pM)

FIG. 5A

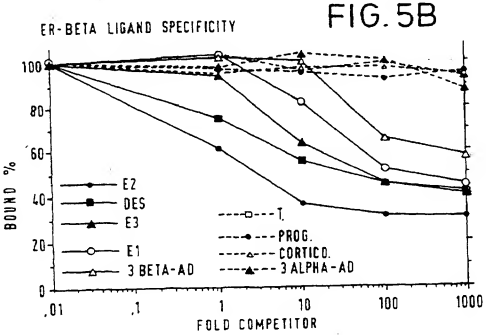

ER-BETA LIGAND SPECIFICITY
BOUND %
100
80
60
40
20
0
.01
.1
1
10
100
1000
FOLD COMPETITOR
E2
DES
E3
E1
3 BETA-AD
T.
PROG.
CORTICD.
3 ALPHA-AD

FIG. 5B

FIG. 5C

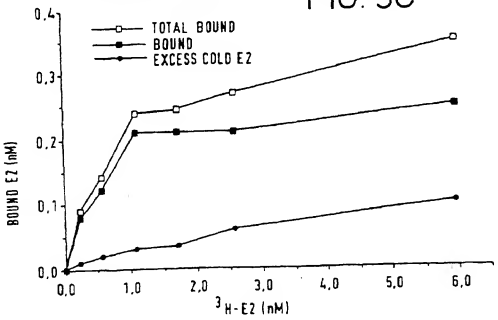

E2 BINDING BY ER-BETA
TOTAL BOUND
BOUND
EXCESS COLD E2
BOUND E2 (nM)
0,4
0,3
0,2
0,1
0,0
0,0
1,0
2,0
3,0
4,0
5,0
6,0
3H-E2 (nM)

FIG. 6

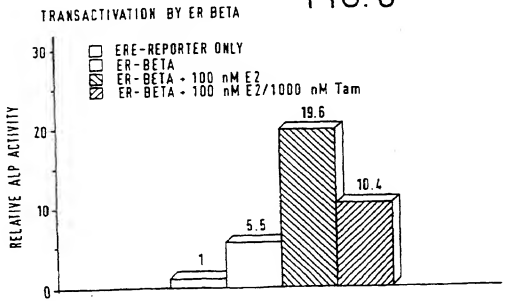

TRANSACTIVATION BY ER BETA
ERE-REPORTER ONLY
ER-BETA
ER-BETA + 100 nM E2
ER-BETA + 100 nM E2/1000 nM Tam
RELATIVE ALP ACTIVITY
30
20
10
0
1
5.5
19.6
10.4

FIG. 7

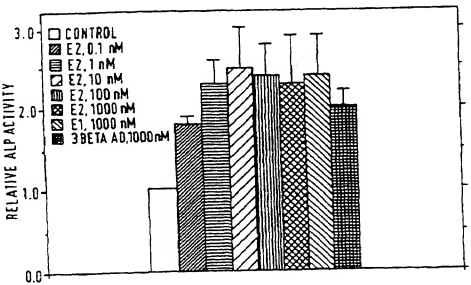
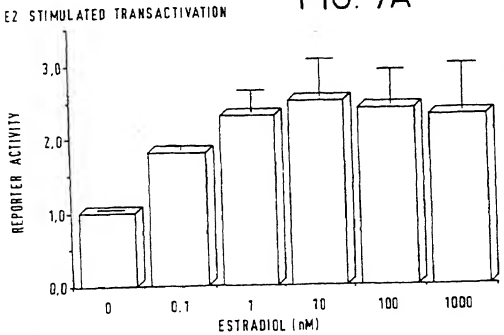

CONTROL
E2, 0.1 nM
E2, 1 nM
E2, 10 nM
E2, 100 nM
E2, 1000 nM
E1, 1000 nM
3 BETA AD, 1000 nM
RELATIVE ALP ACTIVITY
3.0
2.0
1.0
0.0

FIG. 7A


E2 STIMULATED TRANSACTIVATION
REPORTER ACTIVITY
3,0
2,0
1,0
0,0
0
0.1
1
10
100
1000
ESTRADIOL (nM)

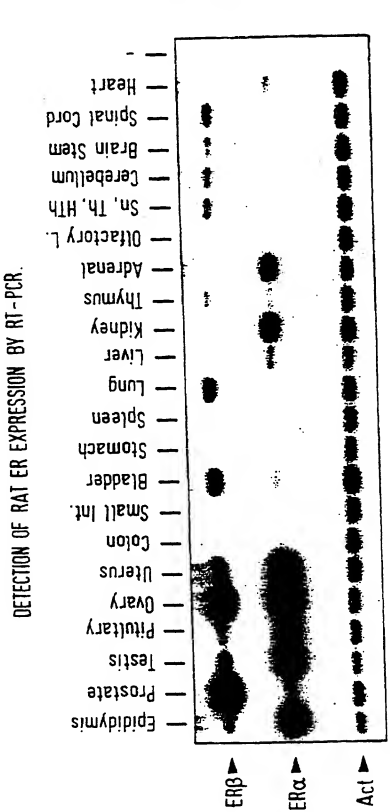

DETECTION OF RAT ER EXPRESSION BY RT-PCR.
Epididymis
Prostate
Testis
Pituitary
Ovary
Uterus
Colon
Small Int.
Bladder
Stomach
Spleen
Lung
Liver
Kidney
Thymus
Adrenal
Olfactory L.
Sn, Th, HTh
Cerebellum
Brain Stem
Spinal Cord
Heart
-
ERβ
ERα
Act

FIG. 8

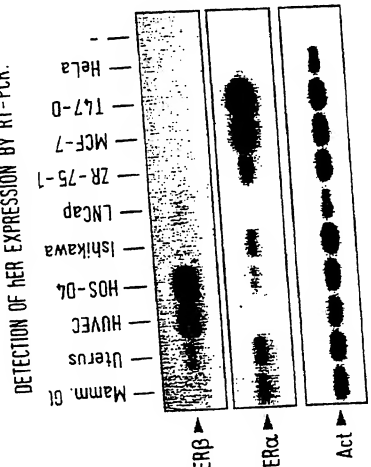

DETECTION OF hER EXPRESSION BY RT-PCR.
Mamm. Gl
Uterus
HUVEC
HOS-OL
Ishikawa
LNCap
ZR-75-1
MCF-7
T47-D
HeLa
-
HERβ
HERα
Act

FIG. 9

FIG. 10A HILL PLOT COMPARING hERα AND rERβ.

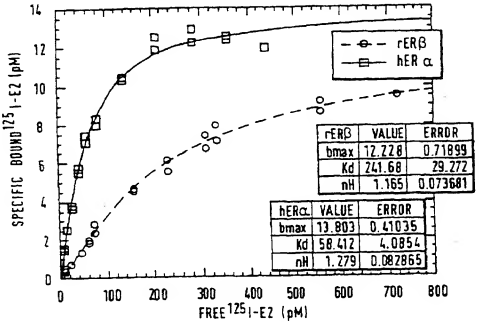


FIG. 10B SCATCHARDPLOT COMPARING hERα AND rERβ.

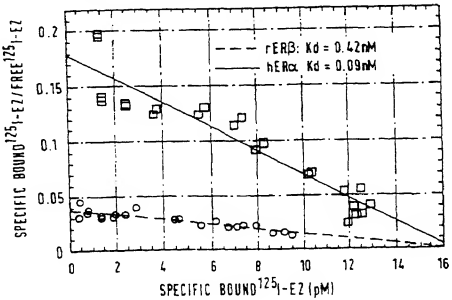


FIG.11A

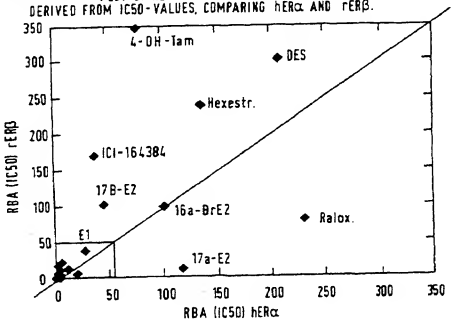


FIG.11B

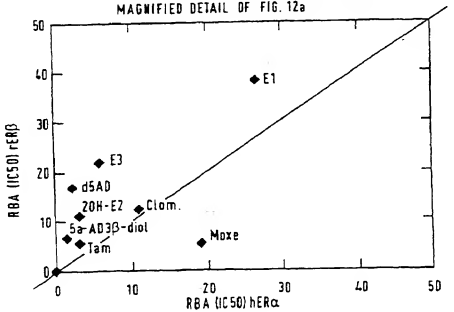


FIG. 12

```
  1  MTFYSPAVMN YSIPSNVTNL EGGPGRQTTS PNVLWPTPGH LSPLVVHRQL
 51  SHLYAEPQKS PWCEARSLEH TLPVNRETLK RKVSGNRCAS PVTGPGSKRD
101  AHFCAVCSDY ASGYHYGVWS CEGCKAFFKR SIQGHNDYIC PATNQCTIDK
151  NRRKSCQACR LRKCYEVGMV KCGSRRERCG YRLVRRQRSA DEQLHCAGKA
201  KRSGGHAPRV RELLLDALSP EQLVLTLLEA EPPHVLISRP SAPFTEASMM
251  MSLTKLADKE LVHMISWAKK IPGFVELSLF DQVRLLESCW MEVLMMGLMW
301  RSIDHPGKLI FAPDLVLDRD EGKCVEGILE IFDMLLATTS RFRELKLQHK
351  EYLCVKAMIL LNSSMYPLVT ATQDADSSRK LAHLLNAVTD ALVWVIAKSG
401  ISSQQQSMRL ANLLMLLSHV RHASNKGMEH LLNMKCKNVV PVYDLLLEML
451  NAHVLRGCKS SITGSECSPA EDSKSKEGSQ NLQSQ*
```

FIG. 13A

```
MAFYSPAVMNYSVPSSTGNLEGGPVRQTASPNVLWPTSGH 40
LSPLATHCQSSLLYAEPQKSPWCEARSLEHTLPVNRETLK 80
RKLGGSGCASPVTSPSTKRDAHFCAVCSDYASGYHYGVWS 120
CEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKNCQACR 160
LRKCYEVGMVKCGSRRERCGYRIVRRQRSASEQVHCLNKA 200
KRTSGHTPRVKELLLNSLSPEQLVLTLLEAEPPNVLVSRP 240
SMPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVELSLL 280
DQVRLLESCWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRD 320
EGKCVEGILEIFDMLLATTARFRELKLQHKEYLCVKAMIL 360
LNSSMYHLATASQEAESSRKLTHLLNAVTDALVWVISKSR 400
ISSQQQSVRLANLLMLLSHVRHISNKGMEHLLSMKCKNVV 440
PVYDLLLEMLNAHTLRGYKSSISGSGCCSTEDSKSKEGSQ 480
NLQSQ. 486
```

FIG. 14A

```
   1  CTATGACATT CTACAGTCCT GCTGTGATGA ATTACAGCAT TCCCAGCAAT
  51  GTCACTAACT TGGAAGGTGG GCCTGGTCGG CAGACCACAA GCCCAAATGT
 101  GTTGTGGCCA ACACCTGGGC ACCTTTCTCC TTTAGTGGTC CATCGCCAGT
 151  TATCACATCT GTATGCGGAA CCTCAAAAGA GTCCCTGGTG TGAAGCAAGA
 201  TCGCTAGAAC ACACCTTACC TGTAAACAGA GAGACACTGA AAAGGAAGGT
 251  TAGTGGGAAC CGTTGCGCCA GCCCTGTTAC TGGTCCAGGT TCAAAGAGGG
 301  ATGCTCACTT CTGCGCTGTC TGCAGCGATT ACGCATCGGG ATATCACTAT
 351  GGAGTCTGGT CGTGTGAAGG ATGTAAGGCC TTTTTTAAAA GAAGCATTCA
 401  AGGACATAAT GATTATATTT GTCCAGCTAC AAATCAGTGT ACAATCGATA
 451  AAAACCGGCG CAAGAGCTGC CAGGCCTGCC GACTTCGGAA GTGTTACGAA
 501  GTGGGAATGG TGAAGTGTGG CTCCCGGAGA GAGAGATGTG GGTACCGCCT
 551  TGTGCGGAGA CAGAGAAGTG CCGACGAGCA GCTGCACTGT GCCGGCAAGG
 601  CCAAGAGAAG TGGCGGCCAC GCGCCCCGAG TGCGGGAGCT GCTGCTGGAC
 651  GCCCTGAGCC CCGAGCAGCT AGTGCTCACC CTCCTGGAGG CTGAGCCGCC
 701  CCATGTGCTG ATCAGCCGCC CCAGTGCGCC CTTCACCGAG GCCTCCATGA
 751  TGATGTCCCT GACCAAGTTG GCCGACAAGG AGTTGGTACA CATGATCAGC
 801  TGGGCCAAGA AGATTCCCGG CTTTGTGGAG CTCAGCCTGT TCGACCAAGT
 851  GCGGCTCTTG GAGAGCTGTT GGATGGAGGT GTTAATGATG GGGCTGATGT
 901  GGCGCTCAAT TGACCACCCC GGCAAGCTCA TCTTTGCTCC AGATCTTGTT
 951  CTGGACAGGG ATGAGGGGAA ATGCGTAGAA GGAATTCTGG AAATCTTTGA
1001  CATGCTCCTG GCAACTACTT CAAGGTTTCG AGAGTTAAAA CTCCAACACA
1051  AAGAATATCT CTGTGTCAAG GCCATGATCC TGCTCAATTC CAGTATGTAC
1101  CCTCTGGTCA CAGCGACCCA GGATGCTGAC AGCAGCCGGA AGCTGGCTCA
1151  CTTGCTGAAC GCCGTGACCG ATGCTTTGGT TTGGGTGATT GCCAAGAGCG
1201  GCATCTCCTC CCAGCAGCAA TCCATGCGCC TGGCTAACCT CCTGATGCTC
1251  CTGTCCCACG TCAGGCATGC GAGTAACAAG GGCATGGAAC ATCTGCTCAA
1301  CATGAAGTGC AAAAATGTGG TCCCAGTGTA TGACCTGCTG CTGGAGATGC
1351  TGAATGCCCA CGTGCTTCGC GGGTGCAAGT CCTCCATCAC GGGGTCCGAG
1401  TGCAGCCCGG CAGAGGACAG TAAAAGCAAA GAGGGCTCCC AGAACCTACA
1451  GTCTCAGTGA
```

FIG. 13B

```
ATGGCATTCTAC AGTCCTGCTGTG ATGAACTACAGT GTTCCCAGCAGC ACCGGTAACCTG GAAGGTGGGCCT   72
GTTCGCCAGACT GCAAGCCCAAAT GTGCTATGGCCA ACTTCTGgACAC CTCTCTCCTTTA GCCACCCACTGC  144
CAATCATCGCTT CTCTATGCAGAA CCTCAAAAGAGT CCTTGGTGTGAA GCAAGATCACTA GAACACACCTTG  216
CCTGTAAACAGA GAGACCCTGAAG AGGAAGCTTGGC GGgAGcgGTTGT GCCAGCCCTGTT ACTAGTCCAAgC  288
ACCAAGAGGGAT GCTCACTTCTGT GCCGTCTGCAGT GATTATGCATCT GGGTATCATTAC GGTGTCTGGTCc  360
TGTGAAGGATGT AAGGCCTTTTTT AAAAGAAGCATT CAAGGACATAAT GACTATATCTGT CCAGCCACGAAT  432
CAGTGTACGATA GACAAGAACCGg CGTAAAAACTGC CAGGCCTGCCGA CTTCGCAAgTGT TACGAAGTAGGA  504
ATGGTCAAGTGT GGATCCAGGAGA GAAAGGTGTGGG TACCGAATAGTA CGAAGACAGAGA AGTGCCAGCGAG  576
CAGGTGCATTGC CTGAACAAAGCC AAGAGAACCAGT GGGCACACACCC CGGGTGAAGGAG CTACTGCTGAAC  648
TCTCTGAGTCCC GAGCAGcTGGTG CTCACCCTGCTG GAAGCTGAGCCA CCCAATGTGCTA GTGAGTCGTCCC  720
AGCATGCCCTTC ACCGAGGCCTCC ATGATGATGTCC CTTACGAAGCTG GCTGACAAGGAA CTGGTGCACATG  792
ATTGGCTGGGCC AAGAAAATCCCT GGCTTTGTGGAG CTCAGCCTGTTG GACCAAGTCCGC CTCTTGGAAAGC  864
TGCTGGATGGAG GTGCTGATGGTG GGGCTGATGTGG CGCTCCATCGAC CACCCCGGCAAG CTCATCTTTGCT  936
CCAGACCTCGtT CTGGACAGGGAT GAgGGGAAGTGC GTgGAAGGGATt cTGGAAATCTTT GaCATGCTCCTG 1008
GCgACGACGGCA CGGTTCCGTGAG TTAAAACTGCAG CACAAAGAATAT CTGTGTGTGAAG GCCATGATTCTC 1080
CTCAACTCCAGT ATGTACCACTTG GCTACCGCAAGC CAGGAAGCAGAG AGTAGCCGGAAG CTGACACACCTA 1152
TTGAACGCAGTG ACAGATGCCCTG GTCTGGGTGATT TCGAAGAGTAGA ATCTCTTCCCAG CAGCAGTCaGTC 1224
CGTCTGGCCAAC CTCCTGATGCTt CTTtCTCATGTC AGGCACATCAGT AAcaAgGGCATG GAACATCTGCTC 1296
AGCATGAAGTGC AAAAATGTGGTC CCGGTGTACGAC CTGCTGCTGGAG ATGCTGAATGCT CACACGCTTCGA 1368
GGGTACAAGTCC TCAATCTCGGGg TCTGgGTGCTGC TCGACAGAGGAC AGTAAGAGCAAA GAGGGCTCCCAG 1440
AACCTCCAGTCT CAGTGA  1458
```

FIG. 14B

CPD	logIC50 hERα	log IC50 rERß	IC50 (nM) hERα	IC50(nM) rERß	Ki (nM) hERα	Ki (nM) rERß	RBA(%), Ki hERα	RBA(%), Ki rERß	RBA(%), IC50 hERα	RBA(%), IC50 rERß
Dihydroepoandrstenedione	-6.31	-6.73	485.29	187.11	245.31	163.33	0.027	0.115	0.027	0.115
Testosterone	-5.00	-5.66	10000.00	2187.76	5750.97	1937.70	0.001	0.010	0.001	0.010
Dihydrotestosterone	-6.36	-7.08	436.52	83.95	220.66	73.28	0.030	0.256	0.030	0.256
4-OH-Estradiol	-8.78	-8.67	1.66	2.14	0.95	1.89	6.934	9.892	7.889	10.037
19-Nor testosterone	-5.82	-7.22	1513.56	60.12	765.10	52.48	0.009	0.357	0.009	0.357
5ß-Androstanedione		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Cyproteronaecetate		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
5-4 androstene 3,17, diona		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Progesterone		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Corticosterone		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Genistein	-8.35	-9.41	4.47	0.39	2.57	0.34	<0.0002%	<0.0002%	<0.0002%	<0.0002%
ß-sitosterol		>-4	>100000	>100000	<0.0002%	<0.0002%	2.576	54.361	2.931	55.157
norethynodrel	-7.53	-7.20	29.51	63.10	14.22	53.09	0.466	0.353	0.444	0.340
norethindrone	-6.50	-5.89	316.23	1288.25	152.32	1083.89	0.043	0.017	0.041	0.017
ß-zearalanol	-8.89	-9.00	1.29	0.99	0.78	0.87	8.457	21.465	10.162	21.657
D-4 androsten 3ß,17ß-diol	-7.33	-7.64	46.77	22.91	23.39	18.71	0.283	1.001	0.280	0.937
dienestrol	-10.03	-10.46	0.09	0.03	0.05	0.03	140.523	661.418	138.995	618.871
Methoxychlor	-5.45	-8.96	3548.13	109.65	1774.07	89.56	0.004	0.209	0.004	0.196
Bisphenol A	-6.41	-7.37	389.05	42.86	194.52	34.84	0.034	0.538	0.034	0.503
Ecdysterone*		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Eudesmine		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Lapidine		>-4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%	<0.0002%	<0.0002%
Tschimgine	-7.18	-6.63	66.07	234.42	40.13	206.47	0.165	0.091	0.198	0.092
Tschimgine	-7.67	-6.45	13.49	354.81	8.19	312.50	0.808	0.060	0.971	0.060
Tschimganidine	-9.10	-9.56	0.79	0.27	0.40	0.24	16.623	78.091	16.623	78.091
Ferutinine	-9.65	-10.12	0.22	0.08	0.14	0.07	46.665	282.307	58.479	284.839
Coumestrol	-9.32	-9.05	0.48	0.90	0.24	0.78	27.530	23.966	27.530	23.966
Naloxidine	-9.88	-9.67	0.13	0.21	0.07	0.19	100.000	100.000	100.000	100.000
16a-Br-E2	-9.44	-8.88	0.36	1.32	0.22	1.16	30.006	16.133	36.058	16.276
17a-E2	-9.68	-9.87	0.21	0.13	0.13	0.12	52.145	157.660	62.661	159.074
17ß-E2										
*)ß-Ecdysone, 20_Hydroxyecdysone										
RBA-values derived from 16a-Br-E2 (100%)										

FIG. 15

EP 0 792 292 B1